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Process for screening glycoform-specific antibodies

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PROCESS FOR SCREENING GLYCOFORM-SPECIFIC ANTIBODIES

The present invention relates to a process for screening glycoform-specific antibodies.

There is currently a growing demand for measurement systems in which antibodies discriminate glycoproteins having a particular glycosylation pattern (*i.e.* discriminate specific glycoforms of a given glycoprotein). Such antibodies are particularly helpful for immunological assays intended for detecting and measuring the concentration of particular glycoforms of a given glycoprotein in body tissues and/or fluids.

For instance, the concentration, glycosylation and/or tissular source of glycoprotein hormones, such as TSH, LH or FSH, may be indicative of certain pathologies (Spencer, C.A. *et al.*, 1995; Spencer, C.A., and Demers, L.M., 2003).

However, several obstacles preclude the obtaining of such antibodies, the most important of which concerns the glycoprotein to be used to screen and/or to elicit the production of antibodies.

It is difficult to obtain glycoproteins with a desired glycosylation pattern, especially with a human-type glycosylation pattern, reliably and in large amounts. Thus, human or animal sources of glycoproteins, provide for very scarce amounts of protein. Moreover, the glycosylation pattern of the glycoproteins originating from animals is relatively different from that of human origin.

Recombinant glycoproteins are not an interesting alternative, in that the cell lines used for producing recombinant glycoproteins are mostly of animal origin. Therefore, these cell lines lead to the obtaining of a glycosylation pattern which markedly differ from the human-type glycosylation pattern. Besides, human cell lines can not produce all the various tissue-specific glycosylated glycoproteins.

Accordingly, research is currently being conducted to genetically design new cellular lines so as to produce glycoproteins carrying particular human type glycosylation patterns. Yet these lines are still not suited for protein production in large quantities and the cell line system is not sufficiently versatile to reproduce the whole array of human glycosylation types.

In another instance, the immunoreactivity of desialylated recombinant TSH produced in CHO cells was studied (Zerfaoui, M., and Ronin, C., 1996). However, the interest of using this single modification to screen glycoform-specific antibodies is dismal because most natural glycoproteins, especially those circulating in blood, are sialylated to a certain extent.

Thus, the present invention aims at:

- providing a new process for screening glycoform-specific antibodies;

- using the screened antibodies for the detection of a given glycoprotein;

- providing a process for isolating specific glycoforms of a given glycoprotein, said glycoforms being used:

  - to screen glycoform-specific epitopes,
  - to replace said glycoprotein to calibrate glycoform-specific immunoassays, or
  - to elicit glycoform-specific antibodies.

The present invention relates to the use of the assessment of the binding between

- antibodies elicited against a first glycoprotein, and
- at least one glycoform of a second glycoprotein, said second glycoprotein being itself a glycoform of the first protein,

wherein said glycoform of the second glycoprotein is selected from a group of glycoforms of the second glycoprotein, each glycoform of said group corresponding to a determined glycosylation state defined by a determined sialylation state, and/or a determined branching state, and/or a determined fucosylation state, provided that said glycosylation state is not uniquely defined by a substantially unsialylated state,

for the screening of glycoform specific antibodies directed against a given glycoform of the second glycoprotein.

Most glycoproteins can be found under several forms which vary from each other by their respective glycan content, their respective protein sequence being essentially similar, *i.e.* the amino acid sequence similarity of each glycoprotein as compared to each other being greater than 90%, preferably greater than 95%, provided that both glycoproteins display the same biological properties; those forms are called glycoforms of a given glycoprotein.

The expression "antibodies elicited against a first glycoprotein" means that said antibodies are obtained after immunization of an animal by said first glycoprotein.

Advantageously those antibodies are monoclonal antibodies.

The glycosylation state of a given glycoprotein corresponds to the number of glycan chains carried by said glycoprotein and to the respective structures of those chains.

As a general rule a given glycoform can be characterized by a determined glycosylation state. However, it is worth noting that due to the remarkable diversity of the various protein glycosylation processes in cells, a given glycoform does not correspond to a unique glycosylation pattern but to a set of several closely related glycosylation patterns which can be accounted for by their average glycan structure. This average glycan structure will be herein understood to correspond to said glycosylation state.

The sialylation state corresponds to the number of sialyl groups which are carried by a given glycoprotein. In human glycoproteins, sialic acid most often corresponds to N-acetylneuraminic acid (NeuAc).

The expression "substantially unsialylated state" refers to a glycosylation pattern essentially lacking sialyl groups.

A glycosylation state is said to be "not uniquely defined by a substantially unsialylated state" if it is either not unsialylated or if its unsialylation is associated with other modification of the glycosylation state.

A glycoform, the glycosylation state of which is uniquely defined by a substantially unsialylated state, is of no relevance in the frame of the present invention since this feature is not a natural glycosylation feature, in particular in human circulation.

The fucosylation state corresponds to the number of fucosyl group which are carried by a given glycoprotein. In human glycoproteins, fucose corresponds to L-deoxygalactose.

The branching state corresponds to the number of β 1,2/4/6 linked N-acetylglucosamine residues established with either of the branched mannose residue of the invariant pentasaccharide of N-linked glycans.

By way of illustration the structure of a typical N-linked glycan chain is represented in Figure 1A.

The glycan chain composition, especially the estimation of NeuAc, of fucose and of the degree of branching, can be assessed by acid hydrolysis of the glycoprotein, followed by derivatization and gas chromatography (Methods on Glycoconjugates: a laboratory manual. Ed: André Verbert, 1995). These data indicate the average composition per glycan chain (*i.e.* for 3 mannose residues).

A detailed glycan structure is obtained after releasing the glycan chains by endoglycosidase treatment and isolating each glycan chain by HPLC, following or not chemical derivatization by a fluorophore group. The molecular size of each species/peak is then resolved by mass spectrometry and its carbohydrate sequence is assessed by glycosidase sequential removal and further analysis of the shift in molecular weight using the same technique.

The present invention also relates to a process for screening glycoform specific antibodies among antibodies elicited against a first glycoprotein, comprising a step of determination of the binding between

- antibodies elicited against a first glycoprotein, and

- at least one glycoform of a second glycoprotein, said second glycoprotein being itself a glycoform of the first protein,

wherein said glycoform of the second glycoprotein is selected from a group of glycoforms of the second glycoprotein, each glycoform of said group corresponding to a determined glycosylation state defined by a determined sialylation state, and/or a determined branching state, and/or a determined fucosylation state, provided that said glycosylation state is not uniquely defined by a substantially unsialylated state

to recover antibodies liable to bind to at least one given glycoform of the second glycoprotein.

According to a particular embodiment, the glycosylation state of the glycoform of the second glycoprotein presents at least one of the following criteria:

- it is essentially more sialylated than said second glycoprotein, or
- it is essentially less sialylated than said second glycoprotein, or
- it is essentially more branched than said second glycoprotein, or
- it is essentially less branched than said second glycoprotein, or
- it is essentially more fucosylated than said second glycoprotein, or
- it is essentially less fucosylated than said second glycoprotein.

The expression "more sialylated" means that the glycan chains of said glycoform contain on average more sialyl groups than the glycan chains of the second glycoprotein do.

The expression "less sialylated" means that the glycan chains of said glycoform contain on average less sialyl groups than the glycan chains of the second glycoprotein do.

The expression "more branched" means that the glycan chains of said glycoform are on average more branched than the glycan chains of the second glycoprotein are.

The expression "less fucosylated" means that the glycan chains of said glycoform are on average less branched than the glycan chains of the second glycoprotein are.

The expression "more fucosylated" means that the glycan chains of said glycoform contain on average more fucosyl groups than the glycan chains of the second glycoprotein do.

The expression "less fucosylated" means that the glycan chains of said glycoform contain on average less fucosyl groups than the glycan chains of the second glycoprotein do.

These glycosylation states can be determined following the above described procedure.

Glycan heterogeneity is known to affect pharmacokinetics of glycoproteins, especially those circulating in blood depending on their sialic acid content. Glycosylation is often controlling biological properties of glycoproteins, such as receptor activation, pharmacokinetics of glycoprotein hormones (TSH, LH, FSH and hCG) and also physicochemical/structural properties, such as solubility, isoelectric point, thermal stability

and resistance to proteolytic degradation. Except for sialic acid, these modifications cannot be attributed to a single monosaccharide, but often results from a steric contribution of the whole glycan chain.

According to another particular embodiment, the binding between at least one of the antibodies elicited against the first glycoprotein and each of the glycoforms of the second glycoprotein which are respectively:

- essentially more sialylated than said second glycoprotein,
- essentially less sialylated than said second glycoprotein,
- essentially more branched than said second glycoprotein,
- essentially less branched than said second glycoprotein,
- essentially more fucosylated than said second glycoprotein, and
- essentially less fucosylated than said second glycoprotein,

is determined.

According to another particular embodiment, the glycosylation state of the glycoform of the second glycoprotein presents at least two of the following criteria:

- it is essentially more sialylated or less sialylated than said second glycoprotein,
- it is essentially more branched or less branched than said second glycoprotein,
- it is essentially more fucosylated or less fucosylated than said second glycoprotein.

These modifications act as a combinatory code to generate a remarkable microheterogeneity onto the polypeptide backbone of the protein, affecting both its physicochemical and biological properties. In the case of glycoprotein hormones, inner glycosylation controls subunit association, immunologically and biologically active conformation, while the signal sulphate/sialic acid governs pulsatility and metabolic clearance because it is under the control of the endocrine hypothalamo-pituitary axis.

"Inner glycosylation" typically refers to the invariant pentasaccharide attached to the polypeptide by a N-glycosidic bond. It is known that fucosylation of this core enhances its hydrophobicity and rigidity and, as a result, may alter the conformation of any peptide area covered by the whole glycan.

According to another particular embodiment, the glycosylation state of the glycoform of the second glycoprotein presents one of the following criteria:

- it is essentially more sialylated and more fucosylated than said second glycoprotein, or
- it is essentially more sialylated and less fucosylated than said second glycoprotein, or
- it is essentially more sialylated and more branched than said second glycoprotein, or
- it is essentially more sialylated and less branched than said second glycoprotein, or

- it is essentially less sialylated and more fucosylated than said second glycoprotein, or
- it is essentially less sialylated and less fucosylated than said second glycoprotein, or
- it is essentially less sialylated and more branched than said second glycoprotein, or
- it is essentially less sialylated and less branched than said second glycoprotein, or
- it is essentially more branched and more fucosylated than said second glycoprotein, or
- it is essentially more branched and less fucosylated than said second glycoprotein, or
- it is essentially less branched and more fucosylated than said second glycoprotein, or
- it is essentially less branched and less fucosylated than said second glycoprotein.

According to another particular embodiment, the glycosylation state of the glycoform of the second glycoprotein presents three of the following criteria:

- it is essentially more sialylated or less sialylated than said second glycoprotein,
- it is essentially more branched or less branched than said second glycoprotein,
- it is essentially more fucosylated or less fucosylated than said second glycoprotein.

These modifications generate a remarkable microheterogeneity onto the polypeptide backbone of the protein, affecting both its physicochemical and biological properties during biosynthesis, intracellular migration, secretion and further action at the target tissue. In the case of glycoprotein hormones, this heterogeneity at different levels also controls subunit association, activation of the target receptor, pulsatility and metabolic clearance because it is under the control of the endocrine hypothalamo-pituitary axis.

According to another particular embodiment, the glycosylation state of the glycoform of the second glycoprotein presents one of the following criteria:

- it is essentially more sialylated, more branched and more fucosylated than said second glycoprotein,
- it is essentially more sialylated, more branched and less fucosylated than said second glycoprotein,
- it is essentially more sialylated, less branched and more fucosylated than said second glycoprotein,
- it is essentially more sialylated, less branched and less fucosylated than said second glycoprotein,
- it is essentially less sialylated, more branched and more fucosylated than said second glycoprotein,
- it is essentially less sialylated, more branched and less fucosylated than said second glycoprotein,

- it is essentially less sialylated, less branched and more fucosylated than said second glycoprotein,
- it is essentially less sialylated, less branched and less fucosylated than said second glycoprotein.

According to a preferred embodiment, the antibodies elicited against the first glycoprotein bind to the second glycoprotein with an affinity equal to or higher than the binding affinity of said antibodies to the first glycoprotein.

The measure of the affinity of an antibody for a given glycoprotein can be done according to Benkirane, M.M., *et al.*, 1987.

According to another preferred embodiment, at least one lectin fractionation of the second glycoprotein is performed to obtain a glycoform of the second glycoprotein of a determined glycosylation state.

By "lectin" is meant a sugar-binding protein of non-immune origin, which agglutinates cells and/or recognize glycoconjugates. Lectins are classified according to the monosaccharide which inhibits the interaction between the lectin and the targeted glycan or which allows the specific elution of a bound glycan from an immobilized lectin column. The expression "lectin fractionation" means that glycoforms are separated according to their binding affinity for a given lectin insolubilized on a matrix and used as a chromatography column.

Certain lectins have a specific affinity towards a given monosaccharide, like mannose/glucose, fucose, galactose/N-acetylgalactosamine, N-acetyl-glucosamine or N-acetylneuraminic acid.

Advantageously sugar-specific lectin fractionation enables to separate glycoproteins according to their content in said sugar.

According to another preferred embodiment, the lectin is selected from the group comprising mannose-specific lectins, such as the ConA or Lentil lectins, fucose-specific lectins, such as the Ulex lectin, gactose-specific lectins, such as ricin, or sialic acid-specific lectins, such as limulin or the *Sambucus nigra* lectin.

Those lectins are commercially available and well known to the man skilled in the art.

According to another preferred embodiment, at least one enzymatic modification of the second glycoprotein is performed to obtain a glycoform of the second glycoprotein of a determined glycosylation state.

By enzymatic modification is meant that one or more carbohydrate groups are added, removed or modified in a given glycoprotein.

According to another preferred embodiment, the enzymatic modification is carried out by an enzyme selected from the group comprising a glycosidase, in particular a neuraminidase or a fucosidase, or a glycosyltransferase, in particular a sialyl transferase or a fucosyl transferase.

A neuraminidase selectively removes sialic acid from glycoproteins.

A sialyl transferase specifically adds sialyl groups onto a glycoprotein.

A fucosidase selectively removes fucose from glycoproteins.

A fucosyl transferase specifically adds fucosyl groups onto a glycoprotein.

According to a particularly preferred embodiment, a glycoform of the second glycoprotein of a determined glycosylation state is obtained by a combination of at least one enzymatic modification of the second glycoprotein and/or of at least one lectin fractionation.

According to another preferred embodiment, a less sialylated glycoform of the second glycoprotein as compared to the second glycoprotein is obtained by neuraminidase treatment of said second glycoprotein.

According to another preferred embodiment, a more sialylated glycoform of the second glycoprotein as compared to the second glycoprotein is obtained by sialytransferase treatment of said second glycoprotein or by neuraminidase treatment followed by sialyltransferase treatment of said second glycoprotein.

According to a preferred embodiment, a less fucosylated glycoform of the second glycoprotein as compared to the second glycoprotein is obtained by lentil fractionation of the second glycoprotein by collecting the fraction which does not bind to lentil and a more fucosylated glycoform of the second glycoprotein as compared to the second glycoprotein is obtained by collecting the fraction which binds to lentil.

According to yet another preferred embodiment, a ConA fractionation of the second glycoprotein is performed by collecting three fractions, A, B, and C, the binding of which to ConA is such that,

- C binds to ConA more strongly than B does, and
- B binds to ConA more strongly than A does,

the branching state of a given fraction being essentially different from the branching state of the other two fractions.

According to a particular embodiment, in a preliminary step, the antibodies to be screened are classified in pools, each pool being characterized in that two antibodies selected from a same pool can not bind to the same glycoprotein at the same time.

The expression "at the same time" means that both antibodies compete for a similar binding site on said glycoform.

According to another particular embodiment, in a first step, said first step preceding the preliminary step defined above, it is checked that the antibodies elicited against the first glycoprotein bind to the second glycoprotein.

Advantageously, a complete process according to the invention comprises the following steps:

- checking that the antibodies elicited against the first glycoprotein bind to the second glycoprotein,
- classifying the antibodies to be screened in pools, each pool being characterized in that two antibodies selected from a same pool can not bind to a same glycoform of the second glycoprotein at the same time,
- determining the binding between
  - said antibodies, and
  - at least one glycoform of the second glycoprotein,

to recover antibodies liable to bind to at least one given glycoform of the second glycoprotein.

According to yet another particular embodiment, the binding of the antibodies to the first glycoprotein, to the second glycoprotein and to the glycoforms of the second glycoproteins is determined by using immunoassays, in particular immunoassay formats using an amplification system for detection, such as an ELISA.

According to a much preferred embodiment, the immunoassay is a sandwich immunoassay, in particular a sandwich ELISA test, comprising the following steps:

- fixing a capture antibody, selected from a pool such as defined above, onto a support,
- contacting a glycoprotein, corresponding to the first glycoprotein, to the second glycoprotein or to the glycoforms of the second glycoprotein, to said capture antibody, to form, if adequate, a capture antibody-glycoprotein binary complex,
- contacting a tracer antibody, selected from a pool such as defined above, provided said pool is different from the one used for the selection of said capture antibody, to said capture antibody-glycoprotein binary complex, to form, if adequate, a capture antibody-glycoprotein-tracer antibody ternary complex,
- detecting the tracer antibody for measuring the number of ternary complexes.

The expression "capture antibody" relates to an antibody which is covalently or non-covalently adsorbed onto a support, a so called solid-phase, such as the wall of a

microtitration well, so as to form a capture antibody-glycoprotein complex bound to the solid phase.

The expression "tracer antibody" relates to an antibody which can bind said glycoprotein once it is linked to the capture antibody, said tracer antibody being coupled to a detection system. For instance, the tracer antibody can be linked to biotin and be detected using an avidin-enzyme complex, such as alkaline phosphatase or peroxidase, which may be used to activate a fluorogenic and/or a chromogenic susbtrate, such as p-nitrophenylphosphate, 5 bromo-4-chloro-3indoylphosphate or 1,2 phenyl-enediamine/hydrogen peroxide using an appropriate buffer. Any other enzymatic reaction which is readable with a microtiter spectrofluorometer or spectrophotometer can also be used.

According to another particular embodiment, the first glycoprotein and the second glycoprotein are similar.

The term "similar" means that according to this embodiment the first and the second glycoprotein are the same, that is, they share the same amino acid sequence and the same glycosylation pattern.

According to this particular embodiment, antibodies are elicited against a given glycoprotein, and then the binding of said antibodies with glycoforms of said glycoprotein is determined.

According to yet another particular embodiment, the first glycoprotein and the second glycoprotein originate from different natural tissues and/or fluids.

Tissues and fluids according to the invention notably encompass endocrine tissues, such as the pituitary gland, placenta or endocrine tumors, or fluids such as blood, plasma, serum or urine for instance.

According to still another particular embodiment, the first glycoprotein originates from a natural tissue and the second glycoprotein is a recombinant protein.

Advantageously, the recombinant glycoprotein is produced in mammalian cell lines, in particular Chinese Hamster Ovary (CHO) or human cell lines.

According to a more particular embodiment, the first glycoprotein originates from a natural tissue and the second glycoprotein is a mutated recombinant protein.

By "mutated recombinant protein" is meant that the sequence of second glycoprotein has been modified, with respect to the sequence of the first glycoprotein, by substitution, insertion or deletion of at least one amino acid, provided said second glycoprotein retains hormone-specific immunoreactivity and biological activity.

According to a further particular embodiment, the first glycoprotein is a N-linked glycoprotein, such as TSH, in particular pituitary TSH, LH, FSH, or placental hCG.

According to a much preferred embodiment, the first glycoprotein is pituitary TSH and the second glycoprotein is a recombinant TSH.

The present invention further relates to the use of a glycosylation-specific antibody as screened by the above defined process, for the binding or the purification of given glycoforms of the second glycoprotein.

In particular the above glycosylation-specific antibodies can be used for immunodetecting a given glycoform of the second glycoprotein or for immunoassaying the concentration of a given glycoform of the second glycoprotein, in samples, in particular human biological samples.

The above glycosylation-specific antibodies can also be used to purify a given glycoform of the second glycoprotein, for instance by using affinity chromatography, from samples derived either from biological samples or from culture media of cells lines producing recombinant glycoproteins.

According to a preferred embodiment, antibodies R2 and/or OCD1 are used for the binding or the purification of TSH circulating in blood of healthy subjects or of patients suffering from thyroid diseases, such as hypothyroidism, or from non-thyroid diseases coupled to altered levels of TSH, such as endocrine tumors, chronic renal failure or non-thyroid illnesses.

The present invention also relates to a kit for assaying specific glycoforms of the first glycoprotein, characterized in that it comprises:

- at least one antibody such as screened according to the above defined process,
- at least one glycoprotein calibrant selected from the group comprising the first glycoprotein, the second glycoprotein, and a given glycoform of the second glycoprotein such as defined above.

The expression "glycoprotein calibrant" refers to a glycoprotein which can be used as a standard for the calibration of said kit.

According to a preferred embodiment, the invention relates to a kit as defined above, for assaying TSH in a biological sample, characterized in that it comprises:

- at least one antibody selected from the group comprising:

  pool I antibodies: B1, B2, S04, S09, R1 and BC27,

  pool II antibodies: OCD1 and R2,

  pool III antibodies: B3 and S06,

as capture antibody, and at least one antibody selected from the group comprising:

pool I antibodies: B1, B2, S04, S09, R1 and BC27,

pool II antibodies: OCD1 and R2,

pool III antibodies: B3 and S06,

as tracer antibody, provided that the capture antibody and the tracer antibody do not belong to a same pool, and

-at least one glycoprotein calibrant which is selected from the group comprising pituitary TSH, substantially unsialylated and/or substantially unfucosylated TSH.

Several immunoassays formats are defined herein depending on the capture antibody / tracer antibody couple used. The choice of a given antibody couple itself depends on the targeted epitopic regions. Three main epitopic region have been defined on TSH in the present invention:

region I, which is recognized by pool I antibodies B1, B2, S04, S09, R1 and BC27,

region II, which is recognized by pool II antibodies OCD1 and R2,

region III, which is recognized by pool III antibodies B3 and S06.

Those antibodies are: OCD1 from Ortho-Clinical Diagnostics, (USA), BC27 commercially available from Beckman-Coulter (USA), R1 and R2 from Roche Diagnostics (G), S04, S06 and S09 commercially available from Seradyn (USA), B1, B2 and B3 from Bayer Diagnostics (G).

According to a preferred embodiment, the invention relates to a kit as defined above, for assaying TSH in a biological sample, characterized in that it comprises:

- at least one antibody selected from the group comprising BC27, S04, B1, S09, R1, and B2, as capture antibody and at least one antibody selected from the group comprising S06 and B3, as tracer antibody, or

- at least one antibody selected from the group comprising S06 and B3 as capture antibody and at least one antibody selected from the group comprising BC27, S04, B1, S09, R1, and B2 as tracer antibody, and

-at least one glycoprotein calibrant which is selected from the group comprising pituitary TSH, substantially unsialylated and/or substantially unfucosylated TSH.

This kit is advantageous for assaying TSH from patients with thyroid dysfunction: this immunoassay format is essentially glycosylation independent, that is, antibody binding is not affected by changes in the glycosylation pattern of the protein antigens to be assayed.

In the above defined kit, it is advantageous to use immunoassay formats I/III or preferably III/I, that is to say formats wherein the capture antibody binds to epitopic region I

and the tracer antibody binds to epitopic region III, or formats wherein the capture antibody binds to epitopic region III and the tracer antibody binds to epitopic region I.

This kit is advantageous for assaying TSH from healthy subjects or from patients suffering from thyroid diseases coupled to altered levels of TSH in that it is satisfactorily calibrated by a pituitary extract and/or IRP pituitary standard.

According to a preferred embodiment, the invention relates to a kit as defined above, for assaying TSH in a biological sample, characterized in that it comprises:

- at least one antibody selected from the group comprising BC27, S04, B1, S09, R1, and B2, as capture antibody,
- at least one antibody selected from the same group comprising OCD1 and R2 as tracer antibody, and
-at least one glycoprotein calibrant which is selected from the group comprising recombinant TSH, and a glycoform of recombinant TSH which is substantially more sialylated and/or less fucosylated than said recombinant TSH.

This kit is advantageous for assaying TSH from healthy subjects or from patients suffering from thyroid diseases, such as hypothyroidism, or from non-thyroid diseases coupled to altered levels of TSH; in this kit the immunoassay involved is of format I/II, which is essentially sialylation dependent, that is, antibody binding to glycoforms of a given TSH having an increased sialylation state as compared to said TSH, is increased.

According to a preferred embodiment, the invention relates to a kit as defined above, for assaying TSH in a biological sample, characterized in that it comprises:

- at least one antibody selected from the group comprising S06 and B3, as capture antibody,
- at least one antibody selected from the same group comprising OCD1 and R2 as tracer antibody, and
-at least one glycoprotein calibrant which is selected from the group comprising a glycoform of recombinant TSH which is substantially more sialylated and/or more fucosylated than said recombinant TSH.

This kit is advantageous for assaying TSH from healthy subjects or from patients suffering from thyroid diseases, such as hypothyroidism, or from non-thyroid diseases coupled to altered levels of TSH; in this kit, the immunoassay involved is of format III/II, which is largely glycosylation dependent in that it may exhibit optimal binding capacity of TSH with altered and/or disease-related glycosylation.

A glycosylation-dependent assay is thereby defined by the use of antibodies which are able to capture the largest array of TSH glycoforms. Such assays can typically accomodate

variation in sialic acid and/or fucose content and, as a result, provide a better estimation of changes in hormone level compared to said glycosylation-independent assay.

Description of the Figures

Figure 1A, Figure 1B, Figure 1C and Figure 1D

Figures 1A to 1D represent the typical glycosylation patterns of the major glycoforms found for N-linked glycoproteins (Figure 1A), human pituitary TSH (pitTSH) (Figure 1B), the recombinant TSH (recTSH) used herein (Figure 1C), and the putative human plasma TSH thought to be the disease-related form of TSH circulating in hypothyroid patient (Figure 1D). Asn-X-Ser/Thr corresponds to the consensus peptide sequence for N-glycosylation. NeuAc corresponds to sialic acid linked to the glycan chain by α2,3 or α2,6 linkages. The grey diamond shapes correspond to fucose, the white squares to N-acetylglucosamine, the grey circles to mannose, the black circles to galactose and the grey triangle to N-acetylgalactosamine.

Figure 2A, Figure 2B, Figure 2C and Figure 2D

Figure 2A-D represent the epitope screening of recTSH as obtained by a sandwich ELISA test, using antibodies BC27, B1, B2, B3, R1, R2, S04, S06, and S09 as capture antibodies and biotinylated-OCD1 as a tracer antibody (Figure 2A), biotinylated-BC27 as a tracer antibody (Figure 2B), biotinylated-S04 as a tracer antibody (Figure 2C), biotinylated-S06 as a tracer antibody (Figure 2D). In figure 2A a negative control is also provided (T(-)).

Figure 3

Figure 3 is a simplified diagrammatic representation of the various epitopic regions (I, II and III) recognized by the BC27, B1, B2, B3, R1, R2, S04, S06, S09 and OCD1 antibodies, as deduced from the epitope screening of recTSH. S06 and B3 recognize overlapping epitopes located in epitopic region III; OCD1 and R2 recognize different epitopes located in epitopic region II; S09, R1 and B2 recognize virtually similar epitopes located in epitopic region I, which are different from the epitope recognized by BC27 and the essentially similar epitopes recognized by S04 and B1, in the same epitopic region I.

Figure 4A, Figure 4B, Figure 4C, Figure 4D, Figure 4E and Figure 4F

Figures 4A to 4F represent sandwich ELISA assays of IRP pitTSH (black diamond shapes), IRP recTSH (black squares) and oversialylated recTSH (white squares). The horizontal axis represents the concentration of TSH (in IU/L) and the vertical axis the OD at 405 nm. Figure 4A corresponds to a I/II format (capture antibody BC27, tracer antibody OCD1), figure 4B corresponds to a II/I format (capture antibody R2, tracer antibody S04), figure 4C corresponds to a I/III format (capture antibody BC27, tracer antibody S06), figure 4D corresponds to a III/I format (capture antibody S06, tracer antibody S04), figure 4E corresponds to a II/III format (capture antibody R2, tracer antibody S06) and figure 4F corresponds to a III/II format (capture antibody S06, tracer antibody OCD1).

Figure 5A, Figure 5B and Figure 5C

Figures 5A-C represent ELISA assays of various TSH preparations varying in sialic acid content. The horizontal axis represents the concentration of TSH (in IU/L) and the vertical axis the OD at 405 nm. The black squares correspond to IRP recTSH, the black triangles to asialo-recTSH, the black diamond shapes to IRP pitTSH, the white squares to oversialylated recTSH (recTSH sialylated with α2,6 sialyltransferase) and the white triangles to resialylated recTSH (asialo-recTSH treated with α2,6 sialyltransferase). Figure 5A represents the results of the S06-BC27 sandwich, figure 5B the results of the BC27-OCD1 sandwich and figure 5C a control measurement of α2,6-linked sialic acid using *Sambucus nigra* (SNA) lectin binding.

Figure 6A and Figure 6B

Figure 6A represents the kinetics of OCD1 binding to TSH preparations in a BC27-OCD1 sandwich ELISA assay. The horizontal axis represents the incubation time with OCD1 in minutes and the vertical axis the OD at 405 nm. The black squares correspond to IRP recTSH, the black triangles to asialo-recTSH, the black diamond shapes to IRP pitTSH, the white squares to oversialylated recTSH (recTSH treated with α2,6 sialyltransferase) and the white triangles to resialylated recTSH (asialo-recTSH treated with α2,6 sialyltransferase).

Figure 6B represents a SNA binding assay of the previous TSH preparation (horizontal axis) as measured by the OD at 405 nm (vertical axis).

Figure 7A and Figure 7B

Figures 7A and 7B represent lectin affinity chromatography profiles for recTSH detected with III/I format (S06-BC27). In figure 7A an unbound fraction (eluted by TBS) and a bound

fraction (eluted by 500 mM α-methylmannopyranoside) are shown. The bound fraction is enriched in fucosylated recTSH as compared to the unbound fraction.

In Figure 7B, three fractions are shown, an unbound fraction (eluted by TBS), a weakly bound fraction (eluted by 10 mM α-methylglucopyranoside) and a firmly bound fraction (eluted by 500 mM α-methylmannopyranoside). The three fractions differ from each other with respect to their respective branching states.

Figure 8A and Figure 8B

Figures 8A and 8B represent sandwich ELISA assays of recTSHs purified by lentil affinity chromatography using the BC27-OCD1 format (figure 8A) and the S06-S04 format (figure 8B). The horizontal axes represent the concentration of TSH (in IU/L) and the vertical axes the OD at 405 nm. The black squares represent IRP recTSH, the black cross IRP pitTSH, the black diamond shapes the lentil unbound fraction of recTSH and the black triangles the lentil bound fraction of recTSH.

Figure 9A and Figure 9B

Figures 9A and 9B represent the kinetics of antibody binding to lentil-chromatography fractions for S06 binding in S06-S04biot assay (figure 9A) and for S04 binding in S06-S04biot assay (figure 9B). The horizontal axes represent the time in minutes and the vertical axes represent the OD at 405 nm. The black crosses represent IRP pitTSH, the black squares recTSH, the black diamond shapes the lentil unbound fraction and the black triangles the lentil bound fraction.

Figure 10A, Figure 10B, Figure 10C and Figure 10D

Figures 10A-D represent the effect of the TSH branching pattern on antibody binding using the BC27-OCD1 (figure 10A), S06-OCD1 (figure 10B), S06-S04 (figure 10C) and BC27-S06 (figure 10D) formats. The horizontal axis represents the concentration of TSH (in IU/L) and the vertical axis the OD at 405 nm. The black diamond shapes correspond to the ConA unbound fraction, the black squares to the weakly bound fraction, the black triangles to the firmly bound fraction, and the black crosses to IRP pitTSH.

Figure 11A and Figure 11B

Figure 11A represents the percentage of oversialylated recTSH (vertical axis) in the unbound fraction (black bars), the weakly bound fractions (white bars) or the firmly bound fractions

(hatched bars) of a ConA fractionation of oversialylated recTSH according to the indicated sandwich ELISA formats.

Figure 11B represents the percentage of oversialylated recTSH (vertical axis) in the unbound fraction (black bars), or the bound fractions (hatched bars) of a lentil fractionation of oversialylated recTSH according to the indicated sandwich ELISA formats.

Figure 12A, Figure 12B and Figure 12C

Figures 12A-C represent the plateau charge (vertical axis, OD at 405 nm) of the indicated sandwich ELISA formats (horizontal axis) for IRP pitTSH (figure 12A), IRP recTSH (figure 12B) and oversialylated recTSH (figure 12C).

Figure 13A and Figure 13B

Figures 13A and 13B represent the ELISA assays of TSH varying in core-fucose content. The horizontal axes represent the volume of lentil fraction tested (in μL) and the vertical axes the OD at 405 nm. Diamonds correspond to the BC27-OCD1 format, squares to S06-S04, and triangles to S06-OCD1. Figure A represents the results obtained with the unbound fraction of lentil chromatography (non fucosylated oversialylated recTSH), and figure B the results obtained with the bound fraction of lentil chromatography (fucosylated oversialylated recTSH).

Examples

Example 1

Immunological characterization of a recombinant glycoprotein: TSH

A panel of monoclonal antibodies elicited against human pituitary TSH (pitTSH) were screened against glycoforms of a preparation of recombinant TSH (recTSH), each glycoform corresponding to a determined glycosylation state defined by a modification of the glycosylation pattern typically in either sialylation, fucosylation or branching.

The following monoclonal antibodies elicited against human pituitary TSH were used: OCD1 (Ortho-Clinical Diagnostics, USA), BC27 (Beckman-Coulter, USA), R1 and R2 (Roche Diagnostics), S04, S06 and S09 (Seradyn, USA), B1, B2 and B3 (Bayer Diagnostics).

Prior to use, the antibodies were biotinylated according to the manufacturer recommendations as follows: 200 μg mAb to be biotinylated, were incubated with 6 μg biotin-7-NHS/DMSO, 2 h at room temperature during gentle stirring. Remaining non reacted biotin-7-NHS was separated by gel filtration on a Sephadex G-25 column previously blocked and washed. Labeled antibody was then eluted with PBS solution, the extinction at 280nm of collected fractions was measured and conjugates concentration determined according to $OD_{280} = \epsilon \times C \times 1$ with extinction coefficient $\epsilon = 1.35$ for mAbs. Eluates containing the conjugate were then pooled.

The recombinant TSH (recTSH) was from Seradyn (USA), and the pituitary TSH (pitTSH) was from Biogenesis (UK).

The following materials and reagents were also used. IRP (International Reference Preparations) standards were ordered at the NISBC (National Institute for Biological Standards and Control, UK). Neuraminidase, asialofetuin, bovine serum albumin (BSA), α-methyl glucopyranoside, α-methylmannopyranoside, cacodylic acid, Triton X-100, CMP-NeuAc, p-NitroPhenylphosphate (p-NPP) and Tween 20 were purchased from SIGMA. SNA-biotin was from Vector Laboratories and the streptavidin-alkaline phosphatase conjugate from Jackson ImmunoResearch Laboratories. Purified rat liver ST6Gal I and biotin labeling kit were from Roche. ConA-Sepharose and Lentil-Sepharose were from Pharmacia Biotech, and

chromatography columns from VWR International as well as microtiter 96-wells plates. All culture reagents were from INVITROGEN-Life Technologies.

The commercial preparation of recTSH was investigated to identify the whole array of glycan structure present in the product. Since many structural features were reported to differ between pitTSH and various preparations of recTSH (Grossmann, M. *et al.*, 1995; Canonne, C. *et al.*, 1995) as well as between the pituitary stock and the circulating hormone in normal subjects or patients with thyroid disorders (Papandreou, M-J., *et al.*, 1993), it was of definite interest to understand how far glycosylation of recTSH may be altered compared to the native hormone. **Figures 1B- 1D** thus represents the first elucidation of the kind applied to TSH.

The glycosylation pattern of the recTSH used was first investigated (Morelle, W., and Michalski, J.C., unpublished results). As expected for a compound expressed in mammalian cells which do not contain the enzymatic machinery for synthesizing the GalNAc-sulfate signal, no such sulfated signal was found in the product. Neither hybrid nor mannose-rich was observed like in the pituitary stock either. Such glycans are often present in pitTSH because the pituitary extract contains immature forms of the hormone. Rather, only serum-type glycans were identified in recTSH, ranging from biantennary to tetraantennary structure, containing inner fucose and terminated in sialic acid. This glycan pattern is in full agreement with the pattern recently observed for gonadotropins produced in CHO cells (Gervais, A., et al., 2003), indicating that the engineered hormone is appropriately glycosylated with complex glycans by the host system but that some control is nevertheless lacking as the final product is quite different in glycosylation from the native hormone. The putative structure of plasma TSH is also represented in **Figure 1D**.

Since the glycans of recTSH cover a wide array of structure totally different from those of the pituitary stock, the glycoforms onto which they are present can be regarded as putative markers of altered glycosylation. Accordingly, the whole recTSH preparation could be used to assess to what extent changes in glycan structure affect antibody recognition. The Inventors therefore screened a panel of 10 different monoclonal antibodies specific for human TSH ($\leq$0.2 % cross-reactivity with gonadotropins) against a highly purified preparation of pitTSH and recTSH. Earlier on, two preparations of recTSH were reported to compete with pitTSH for antibodies directed against pituitary TSH (Kashiwai, T., *et al.*, 1991) indicating that distinct preparations of TSH may indeed share cross-reactivity.

As shown in **Table I**, the antibodies could be classified into three groups, depending on their binding to recTSH to a similar (group A), different (group B) or lower extent (group C) than pitTSH at half-maximal binding capacity. Most antibodies differentially recognized the two preparations, indicating that they target determinants which are under direct or indirect control of glycosylation in the hormone antigen. Since the glycans found in recTSH are typically representative of serum-type glycoproteins in mammals, none of them are antigenic. It results that mAbs are directed against peptidic regions under the steric control of glycan chains. Similar recognition of both preparations was observed in group A (mAb BC27) indicates that at least the underlying epitope is similarly expressed in both preparations. Interestingly, some antibodies like mAbs S06 and R2 displayed increased recognition of recTSH, suggesting that changes in glycosylation may generate epitope expression in glycoforms which are masked or absent in the pituitary preparation. In contrast, the recombinant product is poorly recognized by group C antibodies (mAbs B3 and OCD1), because their epitopes are poorly displayed in the antigen.

Table I. Differential screening of anti-TSH antibodies:

	mAbs	pitTSH	recTSH
Group A *"High binding"*	BC27	++++	++++
Group B *"Medium binding"*	R1	++++	+++
	S09	++	+++
	S06	+	+++
	R2	+	+++
	B1	+++	++
	S04	++	++
	B2	+	++
Group C *"Low binding"*	B3	+	+
	OCD1	+++	+

Since no information is available for any other antigen in the litterature as to whether these findings may be of relevance for improving measurement accuracy, the Inventors were prompted to elucidate the number and location of the common epitopes shared by pitTSH and recTSH which may be useful to design innovative specific assays as the measurand will be clearly identified.

The same panel anti-TSH antibodies were further used to construct different sandwich formats to establish the epitope map of the recombinant hormone. The capture antibody was unmodified while the tracer antibody was biotinylated and the sandwich amplified using the

streptavidin-alkaline phosphatase conjugate. The results were read by measuring the OD at 405 nm. **Figure 2A-C** summarizes the data.

In **Figure 2A**, OCD1 was found to bind recTSH independently of the others and partially overlap with R2. In contrast (**Figure 2B**), mAb BC27 appeared to bind the same epitope as mAbs B2, R1 and S09. The determinant bound by mAbs S04 and B1 should either similar or in close vicinity (**Figure 2C**) while the binding of B3 overlapped with that of S06 (**Figure 2D**). These data allowed the Inventors to delineate within recTSH, 3 distinct epitopes designed as **I, II** and **III** respectively common to both pitTSH and recTSH. The main characteristic feature of these epitopes is that they can be identified by antibodies directed against the natural hormone with high specificity. As shown in **Figure 3,** epitope I is very likely to be the Main Immunogenic Region (MIR) as it is recognized by most antibodies of the panel. Since R2, OCD1 and S06 bound the two TSHs to a different extent, epitopes II and III are likely to exhibit variable expression in the two preparations tested.

Based on these observations, the Inventors therefore decided to construct two novel types of TSH measurements: a first one tentatively aimed at equally measuring forms present in both pit- and recTSHs i.e. independently of changes in their glycosylation pattern. Another one designed as glycosylation-dependent, more likely to measure alteration in the antigen potentially related to a putative onset of a thyroid disorder. To this aim, they therefore designed and tested the all 6 possible assay formats with the 3 epitopes (I, II and III) namely **I/II, II/I, I/III, III/I, II/III, III/II,** based on the calibration with the 2nd IRP pitTSH and the 1st IRP recTSH. In all instances, the recTSH preparation commercially used in this study was found immunologically similar to the IRP recTSH standard. For clarity and because the 2nd IRP pitTSH is most often used in routine measurements, only this calibration has been given in the figures below.

Example 2

I-Single parameter modification of the glycosylation pattern of the glycoprotein antigen

The glycosylation pattern of recTSH was then modified, by either altering its sialylation state, its branching state or its fucosylation state.

I.1. Methods

The sialylation state of recTSH was modified by a neuraminidase treatment, which fully desialylated recTSH, followed by a sialyltransferase treatment by an engineered ST6Gal enzyme, to generate a serum-type sialylation of recTSH.

An *oversialylated recTSH* was also obtained by simply treating recTSH by the truncated form of a sialyltransferase without prior desialylation.

Briefly, the neuraminidase treatment was carried out as follows. 250 ng of recTSH was added to 250 µL neuraminidase buffer (100 mM sodium acetate, 2 mM $CaCl_2$, pH 6.5). Aliquots were incubated with or without 5 mU neuraminidase from *Clostridium perfringens* (Type X) at 30°C overnight, under gentle stirring.

The preferred enzyme is a ST6Gal enzyme as no data in the litterature showed a total coverage of glycoproteins with α2,6 linked sialic acid. Various secreted mutants of improved catalytic efficiency in transferring sialic acid in the α2,6 position have been obtained by deleting the N-terminal of hST6Gal I to position 89 included and a stable CHO-K1 clone producing the soluble form of the Δ1-35 hST6Gal I mutant enzyme has been previously characterized in vitro (Legaigneur, P., et al., 2001). Briefly, the cDNA encoding a truncated Δ1-35/89 form of human ST6Gal I lacking the transmembrane segment was cloned into the pFLAG expression vector containing the preprotrypsinogen signal peptide. This form was stably transfected in CHO-K1 cells (Chinese Hamster Ovary cells). The CHO-K1/Δ 1-35/89 hST6Gal I cell line was grown in DMEM with Glutamax-I medium supplemented with 10% inactivated Foetal Calf Serum, fungizone (2.5µg/mL), gentamycine (50µg/mL), and geneticin (200mg/mL) at 37°C in a 5% CO_2 incubator. The cell culture medium was collected after a 72 h period and further concentrated >15-fold by Centriprep centrifugation. Batches were pooled and the soluble enzyme activity was standardized to a standard calibrator preparation (rat liver ST6Gal I) on asialofetuin as described by Legaigneur, P., *et al.* (2001). Under these conditions, concentrated supernatants were estimated to contain 0.06-0.1 mU/µL and aliquots were stored at -20°C until use.

Sialylation of unsialylated recTSH (*asialo-recTSH*) was carried out in 96-wells microtiter plates. Briefly, the truncated form of hST6Gal I (1.2-2 mU) was added to different amounts of recTSH diluted in PBS containing 0.1% BSA, in a cacodylate buffer (50 mM cacodylate, 0.1% BSA, and 0.1% Triton X-100, pH 6.5) containing 0.9 µg CMP-NeuAc and 2

mM $MnCl_2$ in a final volume of 100 μL, and incubated over a 30 min-4 h period at 37°C. α2,6-linked sialic acid was measured by the sialic acid-specific lectin *Sambucus nigra* agglutinin (SNA) coupled to biotin as previously described (Legaigneur, P., et al., 2001). This modified glycoform of recTSH was named *resialylated recTSH*.

The ConA lectin (concanavalin A) was used to fractionate recTSH according to its branching state. Briefly, 0.5 mL of Con A-Sepharose was poured into a 3 mL disposable column and further equilibrated with 10 mL buffer containing 10 mM Tris-HCl, 150 mM NaCl, and 1 mM $MgCl_2$, $MnCl_2$, and $CaCl_2$ (pH 8.0) as described in Papandreou, M-J., *et al.*, 1993. 5 μg of recTSH were loaded onto the column and allowed to interact with the lectin *at least* for 1 h at room temperature. Unbound recTSH was collected by 10 repeated centrifugations with 1 mL column buffer. Weakly bound and firmly bound fractions were collected using the same procedure and the same buffer with 10 mM α-methylglucopyranoside and with 500 mM α-methylmannopyranoside, respectively. PBS containing 2% BSA was added to stabilize fractionated material. Finally, recTSH concentration was determined by testing 100 μL of each fraction in solide-phase assay.

The Lentil lectin was used to fractionate recTSH according to its fucosylation state. The fractionation proceeded essentially as described for the ConA lectin except that the 10 mM α-methylglucopyranoside elution step was omitted.

I.2. Results

Since measurements of TSH are routinely performed on plasma samples, it was of crucial importance to assess Ab recognition of TSHs that may mimic the glycoforms circulating in blood under normal and pathological conditions. Since sulfated TSH is short-lived (Szkudlinski, M.W., *et al.*, 1995) while sialylated proved to be long-lived in the circulation, the behaviour of recTSHs, with a variable extent of sialylation towards the various formats described above, was first investigated.

To provide a sialylated source of recTSH very similar to plasma glycoproteins, the sialic acid content of the antigen was enzymatically modified by prior treatment by a neuraminidase and/or subsequent addition of α2,6-sialic acid by a recombinant sialyltransferase as described above. This glycosidic bond is not present in recTSH which

only contains α2,3-sialic acid (Morelle, W., and Michalski, J-C., unpublished results). Antibody recognition was investigated by testing five different TSH glycoforms differing in sialylation: pitTSH, recTSH, asialo-recTSH, the so-called oversialylated-recTSH containing additive α2,6-sialic acid in addition to its α2,3-sialic acid content, and resialylated recTSH prepared from asialo-recTSH by adding only α2,6-linked sialic acid. All these glycoforms were assessed in the 6 formats defined above.

Figures 4A-E show the results of the 6 formats applied to IRP pitTSH, IRP recTSH and oversialylated recTSH and **Figures 12A-C**, summarize the plateau charge obtained for IRP pitTSH (**Figure 12A**), IRP recTSH (**Figure 12B**), and oversialylated recTSH (**Figure 12C**) with the various formats. In all formats, the recognition of asialo-recTSH was virtually identical to recTSH and for sake of clarity has not been included in Figure 12.

The S06/BC27 pairing (format III/I) displayed an equal binding of TSH glycoforms similar to 2nd IRP pitTSH and was thus found sialylation-independent (**Figure 5A**). Other formats based on tracer antibodies targeting epitope I behaved very similarly indicating that the Main Immunogenic Region (MIR) is not significantly affected by such altered glycosylation. This indicates that these formats bind and estimate glycoforms expressing the same epitope as in the pituitary IRP standard, independently of changes in terminal glycosylation.

In contrast, testing the BC27/OCD1 pairing (format I/II) revealed higher binding of sialylated TSH glycoforms, and thus exhibited a typical sialylation-dependent behaviour (**Figure 5B**). An increased binding with antigen further modified by α2,6-sialylation compared to the α2,3 linked sialic acid was also evidenced. The presence of α2,6-linked sialic acid in oversialylated glycoforms added by the recombinant α2,6-sialyltransferase was controlled by SNA binding (**Figure 5C**) and mass spectrometry (data not shown).

To better understand the basis of such an increase in antibody binding, antibody recognition was analyzed as a function of time (**Figure 6A**) and the data obtained with the BC27/OCD1 pairing were confirmed with the tracer antibody *i.e.* that recognition of mAb OCD1 heavily relies on the presence of sialic acid. The contents in α2,6-linked sialic acid for the enzymatically engineered glycoforms were also controlled and an enhanced signal after α2,6-sialylation (**Figure 6B**) was detected, indicating that both the oversialylated and

resialylated TSHs reached virtual completion in sialic acid as previously reported (Legaigneur et al., 2001). This demonstrated that increasing the extent of sialylation in TSH allowed a better recognition of the array of TSH glycoforms: virtually, antibody binding to resialylated TSH is 2-fold that of the pitTSH calibrant at equilibrium. This also suggested that among glycoforms, some lack antibody recognition because epitope II is poorly expressed as a result of a low content in sialic acid.

These experiments demonstrated that using a format equally efficient in binding pitTSH and recTSH may result to a 100 % lower binding compared to an assay capable to bind sialylated TSH. Since most circulating forms to be measured in blood were previously demonstrated to be enriched in sialic acid, these data therefore strengthen the importance of having a sialylated calibrator for measuring plasma TSH level under most pathophysiological circumstances. At present, pituitary extracts are most often used for calibrating commercial kits because no plasma TSH can be isolated in sufficient amount from blood. As a result, the current calibrator is not representative of the circulating antigen to be measured. Under these conditions, TSH level is likely to be overestimated as the nature of its glycosylation significantly differs from the pituitary antigen, especially in primary hypothyroidism.

To further investigate what level of glycan heterogeneity may affect TSH immunodetection and possibly deduce what could be the best match between the antibodies and the calibrant, fractions of recTSH, the glycosylation state of which differed, were isolated by affinity chromatography on lectins. This well established chromatography is known to discriminate glycans according to their degree of branching for Con A fractions or their content in inner fucose for the Lentil fractions. Each fraction is supposed to share common structural features but can still contain terminal microheterogeneity. Sialylation does not affect the isolation.

Lentil-lectin affinity chromatography showed that 48.4 ± 3.3 % (±SD) of recTSH glycoforms contained no fucose residue and 51.6 ± 3.3 % had at least one internal fucose among the three N-glycans present on the molecule (**Figure 7A** and **Table II**). This allowed us to isolate lentil-unbound (L-UB) fractions (not fucosylated) and lentil-bound (L-B) fractions (with fucose).

Alternatively, binding of recTSH to the Con A column was distributed in three fractions: unbound, weakly bound and firmly bound (**Figure 7B** and **Table II**). Most recombinant TSHs were retained on Con A [unbound, 15.7 ± 5.9 % (±SD); weakly bound, 29.1 ± 2.9 %; firmly bound, 55.2 ± 7.6 %]. The three fractions were designed as Con A-Unbound (UB), weakly bound (WB) and firmly bound (FB). As in **Figures 5 and 6**, all assays were calibrated with the 2nd IRP pitTSH and 1st IRP recTSH standards.

Table II. Isolation of recTSH fractions based on lectin chromatography:

Lectin-chromatography	Fractions	
Lentil	L-UB	48.4 ± 3.3 %
	L-B	51.6 ± 3.3 %
ConA	ConA-UB	15.7 ± 5.9 %
	ConA-WB	29.1 ± 2.9 %
	ConA-FB	55.2 ± 7.6 %

Results are expressed in % and determined with the S06-BC27 assay.

To study the influence of inner fucosylation, we compared the behaviour of mAbs with fucosylated and non fucosylated TSHs separated by Lentil chromatography to the whole preparation of recTSH and in format calibrated with IRP pitTSH.

The use of the BC27/OCD1 pairing (format I/II) revealed a binding of L-UB and L-B fractions very similar to the crude preparation and pitTSH standard, in that it poorly recognizes the fucosylated forms (**Figure 8A**). In contrast, S06/S04 pairing (format III/I) displayed not only a higher binding of than the IRP standard with an improved recognition of fucosylated glycoforms (**Figure 8B**). A two-fold difference was observed at the EC_{50}, indicating that inner fucosylation increased Ab recognition. It was concluded that this sandwich was not dependent on TSH fucosylation in contrast to the I/II format.

To identify which epitope was fucosylation-dependent, we carried out the kinetics of binding of the capture (mAbs S06) and tracer (mAb S04) antibodies independently. S06 binding was found to display variable binding to the various TSHs upon time whereas mAb S04 showed no discrimination towards the fucose content of TSH glycoforms (**Figure 9**). This allowed us to assign epitope III as being under the control of core fucosylation. It is conceivable that increasing inner fucosylation in TSH may alter expression of this epitope since the content of the recombinant product is high. This also opens the possibility that under primary hypothyroidism, increasing fucosylation of blood TSH may alter TSH measurements

progressively depending on the antibodies used in the assay and results in discordant measurements among kits which contain different combination of Abs.

Since plasma TSH was observed to be largely unretained on ConA chromatography compared to pitTSH standard under normal or pathological conditions (Papandreou, M-J., *et al.*, 1993), the Inventors also paid special attention to the binding of antibodies towards TSHs isolated by this lectin.

Like in the experiments described above, the three TSH fractions against all the formats calibrated against the 2nd IRP pitTSH were tested. In most instances, the respective distribution among Ubd vs WB vs FB fractions was differentially estimated by the various formats while the total recovery was similar. As shown in **Figures 10A-10D**, no difference in dose-dependent binding among the 3 TSH fractions could be noticed among 4 formats, with most of them displaying a significant increase in binding capacity compared to the pitTSH standard. It can be concluded that branching per se does not significantly influence the recognition of any of the 3 epitopes. These data nevertheless further support the proposal that a combination of certain antibodies allowed better binding than others depending on the calibrant used.

As a result, it is proposed that the choice of epitope for the capture antibody should be associated with the use of the calibrant, both being critical for the final measurement of TSH (**Figures 12A-12C**). Then, the choice of a given tracer antibody will further improve the overall quantitation depending whether or not it recognizes an epitope under the control of sialylation. Basically 3 groups of formats can be designed:

1-Formats I/III or III/I:

The MIR determinant can be used to capture glycoforms sharing the highest similarity with pitTSH (glycoprotein 1). Because this latter is recognized by most of the antibodies commercially available, epitope I offers a variety of possibilities for commercial use to construct formats which are poorly sensitive to changes in glycosylation. Epitope III (format I/III) is acceptable to be used as tracer epitope in that it fully accommodates changes in fucosylation. This sandwich format will be best calibrated with pitTSH and other unsialylated/poorly sialylated and poorly fucosylated glycoforms of pit/recTSH like Lentil-unbound fractions.

2-Formats I/II:

Epitope II (format I/II) allows a definite better recognition of TSHs enriched in sialic acid. It should be used in conjunction with a sialylated calibrant to allow satisfactory estimation, especially at low levels of TSH.

When associated to a capture antibody directed against epitope I, the calibrant should also contain a low content in core fucosylation to approach a quantitative measurement of the antigen. As a result the best calibrant would be the Lentil-unbound fraction of over/resialylated recTSH.

3-Formats III/II:

Selecting antibodies targeting epitope III definitely permits to capture the highest amount of glycoforms independently of the nature of the calibrant (pitTSH vs. recTSH) and of the extent of sialylation of the sample measured (recTSH vs. oversialylated recTSH). Using epitope II further increases the maximal binding capacity of the assay. The best estimation will be provided by using a highly sialylated and highly fucosylated calibrant like the Lentil-bound fraction of over/resialylated recTSH.

Since the expression of the 3 relevant epitopes are each under a differential control of TSH glycosylation state, the above proposal is also intended to solve the discordances observed so far among TSH measurements. To allow satisfactory detection of altered TSH enriched in sialic acid and fucose, epitope III will be better used as capter epitope to optimize binding of fucosylated TSHs upon the onset of hypothyroidism. Epitope II as tracer to allow the highest detection of all the forms for which the extent of sialylation has been increased as the disease develops. Since the alteration of TSH glycosylation in the whole panel of thyroid disorders is still unknown, this format appears also best suited for the diagnostic and the follow up of these diseases.

Example 3

Multiple parameter modification of the glycosylation pattern

So as to obtain glycoforms of recTSH for which two or more of the glycosylation parameters are altered, successive modifications of recTSH were carried out by enzymatic treatment and/or chromatographic fractionation, according to the methods already described.

In a first instance, oversialylation of recTSH followed by ConA chromatography was performed. The results of the various immunoassays formats according to the invention are shown in **Figure 11A**.

In another instance, oversialylation of recTSH followed by lentil chromatography was performed. The results obtained for the various immunoassays formats according to the invention are shown in **Figure 11B**.

In still another instance, sialylation was first performed on recTSH, followed by a ConA chromatography and then by a lentil chromatography of the ConA unbound fraction. Our results showed that the oversialylated ConA unbound fraction is not retained on lentil.

Example 4

Selection of antibodies directed against a recombinant TSH

Any of the recombinant TSH preparation or fraction used in the preceding examples can be administered to mice in order to produce several monoclonal antibodies specific for TSH according to the general procedure described by Kohler, G., and Milstein, C. (1975).

The anti-TSH monoclonal antibodies can then be screened against any of the glycoforms of any recTSH fractions or preparations described in Examples 2 and 3.

Several antibodies are obtained which preferentially recognize either the oversialylated or the resialylated glycoforms of recTSH as compared to recTSH itself. Those antibodies are useful for the detection of the putative forms of TSH circulating in blood of hypothyroid patients, patients with non-thyroid illnesses, or TRH-treated patients. Such forms contain highly branched and sialylated chains as reported in Papandreou *et al.*, 1993. Occasionally, as for hypothyroid TSH, they also have altered core fucosylation. Such disease-related glycoforms are best measured with the III/II format calibrated with enzymatically resialylated recTSH fractions. In contrast, low or subnormal TSH levels, as in euthyroid subjects, are better estimated by glycosylation-independent formats calibrated with either pitTSH or recTSH such as the I/III or III/I format. Preferably, the I/II format may show a lower sensitivity threshold, provided that binding properties of the antibodies and calibration with pit/recTSH are further optimized better suited for daily use of these assays which may be quite far from equilibrium.

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CLAIMS

1. The use of the assessment of the binding between

- antibodies elicited against a first glycoprotein, and
- at least one glycoform of a second glycoprotein, said second glycoprotein being itself a glycoform of the first protein,

wherein said glycoform of the second glycoprotein is selected from a group of glycoforms of the second glycoprotein, each glycoform of said group corresponding to a determined glycosylation state defined by a determined sialylation state, and/or a determined branching state, and/or a determined fucosylation state, provided that said glycosylation state is not uniquely defined by a substantially unsialylated state,

for the screening of glycoform specific antibodies directed against a given glycoform of the second glycoprotein.

2. A process for screening glycoform specific antibodies among antibodies elicited against a first glycoprotein, comprising a step of determination of the binding between

- antibodies elicited against a first glycoprotein, and
- at least one glycoform of a second glycoprotein, said second glycoprotein being itself a glycoform of the first protein,

wherein said glycoform of the second glycoprotein is selected from a group of glycoforms of the second glycoprotein, each glycoform of said group corresponding to a determined glycosylation state defined by a determined sialylation state, and/or a determined branching state, and/or a determined fucosylation state, provided that said glycosylation state is not uniquely defined by a substantially unsialylated state,

to recover antibodies liable to bind to at least one given glycoform of the second glycoprotein.

3. A process according to claim 2, wherein the glycosylation state of the glycoform of the second glycoprotein presents at least one of the following criteria:

- it is essentially more sialylated than said second glycoprotein, or
- it is essentially less sialylated than said second glycoprotein, or
- it is essentially more branched than said second glycoprotein, or
- it is essentially less branched than said second glycoprotein, or
- it is essentially more fucosylated than said second glycoprotein, or
- it is essentially less fucosylated than said second glycoprotein.

4. A process according to claim 2 or 3, wherein the binding between at least one of the antibodies elicited against the first glycoprotein and each of the glycoforms of the second glycoprotein which are respectively:

- essentially more sialylated than said second glycoprotein,
- essentially less sialylated than said second glycoprotein,
- essentially more branched than said second glycoprotein,
- essentially less branched than said second glycoprotein,
- essentially more fucosylated than said second glycoprotein, and
- essentially less fucosylated than said second glycoprotein,

is determined.

5. A process according to claim 2 or 3, wherein the glycosylation state of the glycoform of the second glycoprotein presents at least two of the following criteria:

- it is essentially more sialylated or less sialylated than said second glycoprotein,
- it is essentially more branched or less branched than said second glycoprotein,
- it is essentially more fucosylated or less fucosylated than said second glycoprotein.

6. A process according to claim 5, wherein the glycosylation state of the glycoform of the second glycoprotein presents one of the following criteria:

- it is essentially more sialylated and more fucosylated than said second glycoprotein, or
- it is essentially more sialylated and less fucosylated than said second glycoprotein, or
- it is essentially more sialylated and more branched than said second glycoprotein, or
- it is essentially more sialylated and less branched than said second glycoprotein, or
- it is essentially less sialylated and more fucosylated than said second glycoprotein, or
- it is essentially less sialylated and less fucosylated than said second glycoprotein, or
- it is essentially less sialylated and more branched than said second glycoprotein, or
- it is essentially less sialylated and less branched than said second glycoprotein, or
- it is essentially more branched and more fucosylated than said second glycoprotein, or
- it is essentially more branched and less fucosylated than said second glycoprotein, or
- it is essentially less branched and more fucosylated than said second glycoprotein, or
- it is essentially less branched and less fucosylated than said second glycoprotein.

7. A process according to claim 2, 3 or 5, wherein the glycosylation state of the glycoform of the second glycoprotein presents three of the following criteria:

- it is essentially more sialylated or less sialylated than said second glycoprotein,
- it is essentially more branched or less branched than said second glycoprotein,
- it is essentially more fucosylated or less fucosylated than said second glycoprotein.

8. A process according to claim 7, wherein the glycosylation state of the glycoform of the second glycoprotein presents one of the following criteria:

- it is essentially more sialylated, more branched and more fucosylated than said second glycoprotein,
- it is essentially more sialylated, more branched and less fucosylated than said second glycoprotein,
- it is essentially more sialylated, less branched and more fucosylated than said second glycoprotein,
- it is essentially more sialylated, less branched and less fucosylated than said second glycoprotein,
- it is essentially less sialylated, more branched and more fucosylated than said second glycoprotein,
- it is essentially less sialylated, more branched and less fucosylated than said second glycoprotein,
- it is essentially less sialylated, less branched and more fucosylated than said second glycoprotein,
- it is essentially less sialylated, less branched and less fucosylated than said second glycoprotein.

9. A process according to any of claims 2 to 8, wherein the antibodies elicited against the first glycoprotein bind to the second glycoprotein with an affinity equal to or higher than the binding affinity of said antibodies to the first glycoprotein.

10. A process according to any of claims 2 to 9, wherein at least one lectin fractionation of the second glycoprotein is performed to obtain a glycoform of the second glycoprotein of a determined glycosylation state.

11. A process according to claim 10, wherein the lectin is selected from the group comprising mannose-specific lectins, such as the ConA or Lentil lectins, fucose-specific lectins, such as the Ulex lectin, gactose-specific lectins, such as ricin, or sialic acid-specific lectins, such as the limulin or Sambucus nigra lectin.

12. A process according to any of claims 2 to 11, wherein at least one enzymatic modification of the second glycoprotein is performed to obtain a glycoform of the second glycoprotein of a determined glycosylation state.

13. A process according to claim 12, wherein the enzymatic modification is carried out by an enzyme selected from the group comprising a glycosidase, in particular a neuraminidase or a fucosidase, or a glycosyltransferase, in particular a sialyl transferase or a fucosyl transferase.

14. A process according to any of claims 2 to 13, wherein a glycoform of the second glycoprotein of a determined glycosylation state is obtained by a combination of at least one enzymatic modification of the second glycoprotein and/or of at least one lectin fractionation.

15. A process according to any of claims 2 to 14, wherein a less sialylated glycoform of the second glycoprotein as compared to the second glycoprotein is obtained by neuraminidase treatment of said second glycoprotein.

16. A process according to any of claims 2 to 15, wherein a more sialylated glycoform of the second glycoprotein as compared to the second glycoprotein is obtained by sialytransferase treatment of said second glycoprotein or by neuraminidase treatment followed by sialyltransferase treatment of said second glycoprotein.

17. A process according to any of claims 2 to 16, wherein a less fucosylated glycoform of the second glycoprotein as compared to the second glycoprotein is obtained by lentil fractionation of the second glycoprotein by collecting the fraction which does not bind to lentil and a more fucosylated glycoform of the second glycoprotein as compared to the second glycoprotein is obtained by collecting the fraction which binds to lentil.

18. A process according to any of claims 2 to 17, wherein a ConA fractionation of the second glycoprotein is performed by collecting three fractions, A, B, and C, the binding of which to ConA is such that,

- C binds to ConA more strongly than B does, and
- B binds to ConA more strongly than A does,

the branching state of a given fraction being essentially different from the branching state of the other two fractions.

19. A process according to any of claims 2 to 18, wherein, in a preliminary step, the antibodies to be screened are classified in pools, each pool being characterized in that two antibodies selected from a same pool can not bind to the same glycoprotein at the same time.

20. A process according to any of claims 2 to 19, wherein in a first step, said first step preceding the preliminary step of claim 19, it is checked that the antibodies elicited against the first glycoprotein bind to the second glycoprotein.

21. A process according to any of claims 2 to 20, wherein the binding of the antibodies to the first glycoprotein, to the second glycoprotein and to the glycoforms of the second glycoproteins is determined by using immunoassays, in particular immunoassay formats using an amplification system for detection, such as an ELISA.

22. A process according to claim 21, wherein the immunoassay is a sandwich immunoassay, in particular a sandwich ELISA test, comprising the following steps:

- fixing a capture antibody, selected from a pool such as defined in claim 19, onto a support,
- contacting a glycoprotein, corresponding to the first glycoprotein, to the second glycoprotein or to the glycoforms of the second glycoprotein, to said capture antibody, to form, if adequate, a capture antibody-glycoprotein binary complex,
- contacting a tracer antibody, selected from a pool such as defined in claim 19, provided said pool is different from the one used for the selection of said capture antibody, to said capture antibody-glycoprotein binary complex, to form, if adequate, a capture antibody-glycoprotein-tracer antibody ternary complex,
- detecting the tracer antibody for measuring the number of ternary complexes.

23. A process according to any of claims 2 to 22, wherein the first glycoprotein and the second glycoprotein are similar.

24. A process according to any of claims 2 to 22, wherein the first glycoprotein and the second glycoprotein originate from different natural tissues and/or fluids.

25. A process according to any of claims 2 to 22, wherein the first glycoprotein originates from a natural tissue and the second glycoprotein is a recombinant protein.

26. A process according to any of claims 2 to 25, wherein the first glycoprotein is a N-linked glycoprotein, such as TSH, in particular pituitary TSH, LH, FSH, or placental hCG.

27. A process according to any claim 25 or 26, wherein the first glycoprotein is pituitary TSH and the second glycoprotein is a recombinant TSH.

28. The use of a glycosylation-specific antibody as screened by the process according to any of claims 2 to 27, for the binding or the purification of given glycoforms of the second glycoprotein.

29. The use according to claim 28, of antibodies R2 and/or OCD1 for the binding or the purification of TSH circulating in blood of healthy subjects or of patients suffering from thyroid diseases, such as hypothyroidism, or from non-thyroid diseases coupled to altered levels of TSH, such as endocrine tumors, chronic renal failure or non-thyroid illnesses.

30. A kit for assaying specific glycoforms of the first glycoprotein, characterized in that it comprises:
- at least one antibody such as screened according to the process of any of claims 2 to 27,
- at least one glycoprotein calibrant selected from the group comprising the first glycoprotein, the second glycoprotein, and a given glycoform of the second glycoprotein such as defined in any of claims 1 to 27.

31. A kit according to claim 30, for assaying TSH in a biological sample, characterized in that it comprises:

- at least one antibody selected from the group comprising BC27, S04, B1, S09, R1, and B2, as capture antibody and at least one antibody selected from the group comprising S06 and B3, as tracer antibody, or

- at least one antibody selected from the group comprising S06 and B3 as capture antibody and at least one antibody selected from the group comprising BC27, S04, B1, S09, R1, and B2 as tracer antibody, and

-at least one glycoprotein calibrant which is selected from the group comprising pituitary TSH, substantially unsialylated and/or substantially unfucosylated TSH.

32. A kit according to claim 30, for assaying TSH in a biological sample, characterized in that it comprises:

- at least one antibody selected from the group comprising BC27, S04, B1, S09, R1, and B2, as capture antibody,

- at least one antibody selected from the same group comprising OCD1 and R2 as tracer antibody, and

-at least one glycoprotein calibrant which is selected from the group comprising recombinant TSH, and a glycoform of recombinant TSH which is substantially more sialylated and/or less fucosylated than said recombinant TSH.

33. A kit according to claim 30, for assaying TSH in a biological sample, characterized in that it comprises:

- at least one antibody selected from the group comprising S06 and B3, as capture antibody,

- at least one antibody selected from the same group comprising OCD1 and R2 as tracer antibody, and

-at least one glycoprotein calibrant which is selected from the group comprising a glycoform of recombinant TSH which is substantially more sialylated and/or more fucosylated than said recombinant TSH.

ABSTRACT

PROCESS FOR SCREENING GLYCOFORM-SPECIFIC ANTIBODIES

The present invention relates to the use of the assessment of the binding between

- antibodies elicited against a first glycoprotein, and
- at least one glycoform of a second glycoprotein, said second glycoprotein being itself a glycoform of the first protein,

wherein said glycoform of the second glycoprotein is selected from a group of glycoforms of the second glycoprotein, each glycoform of said group corresponding to a determined glycosylation state defined by a determined sialylation state, and/or a determined branching state, and/or a determined fucosylation state, provided that said glycosylation state is not uniquely defined by a substantially unsialylated state,

for the screening of glycoform specific antibodies directed against a given glycoform of the second glycoprotein.

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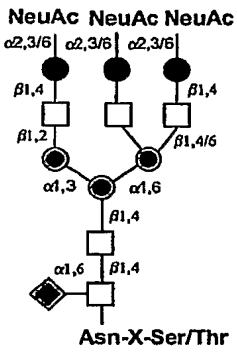
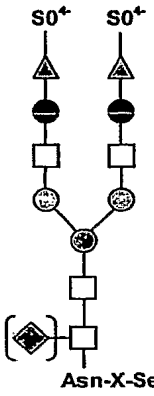


Figure 1A



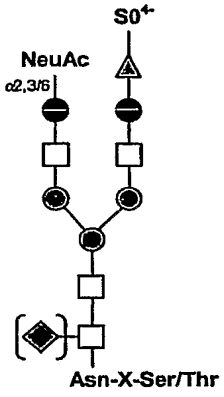
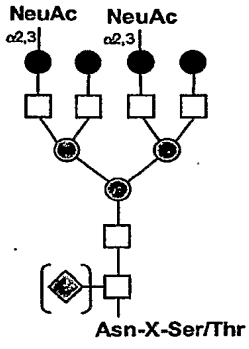
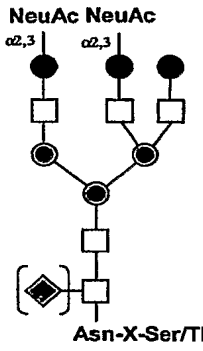


Figure 1B





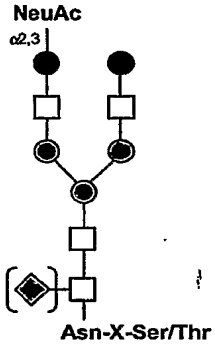


Figure 1C

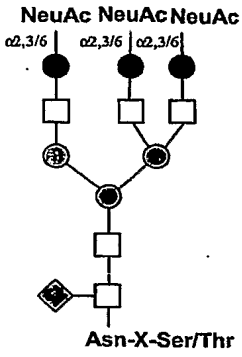


Figure 1D

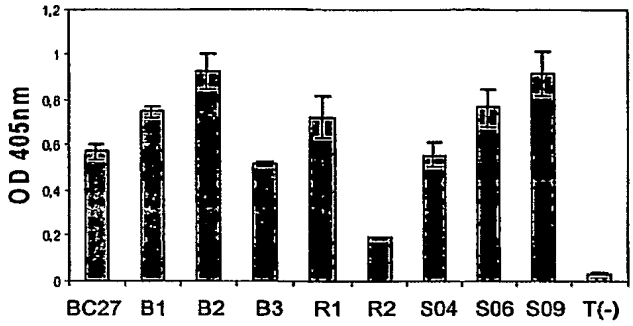


Figure 2A

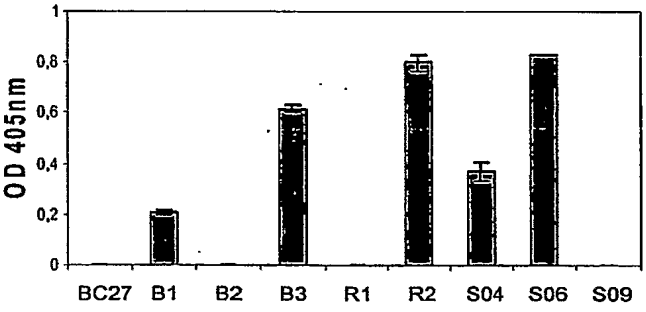


Figure 2B

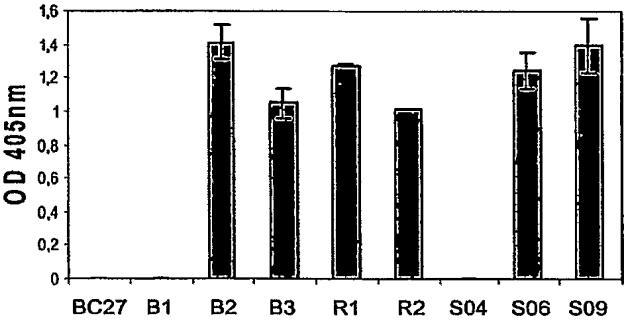


Figure 2C

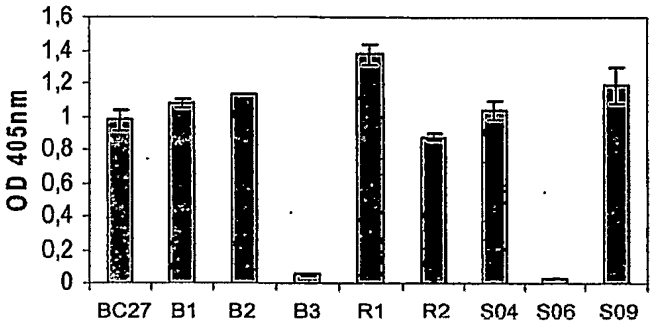


Figure 2D

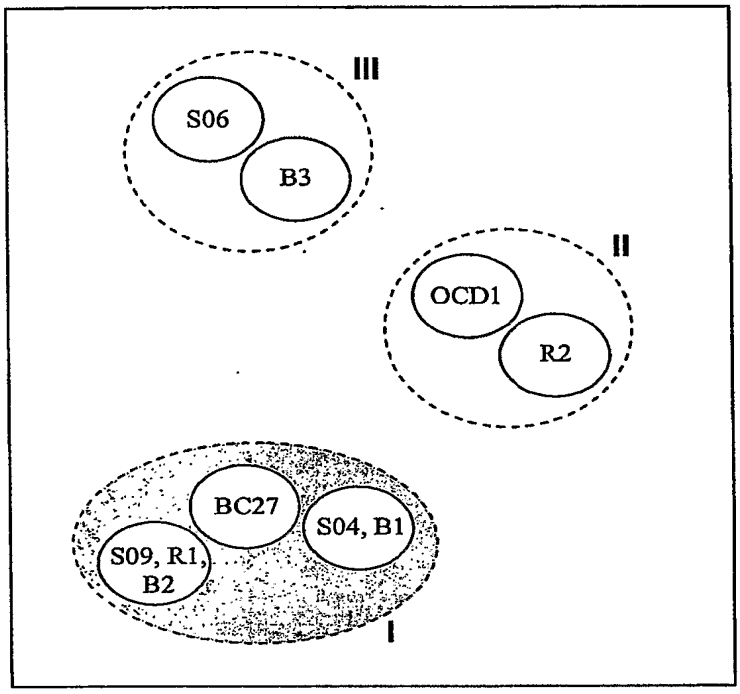


Figure 3

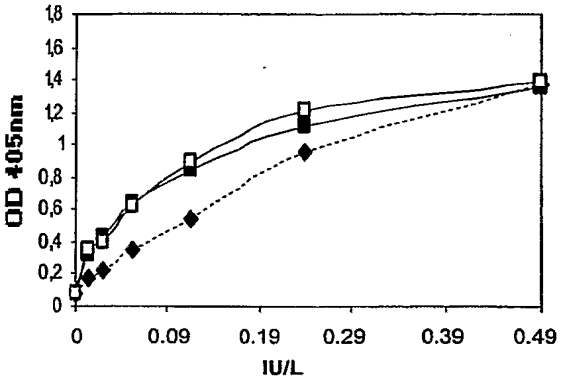
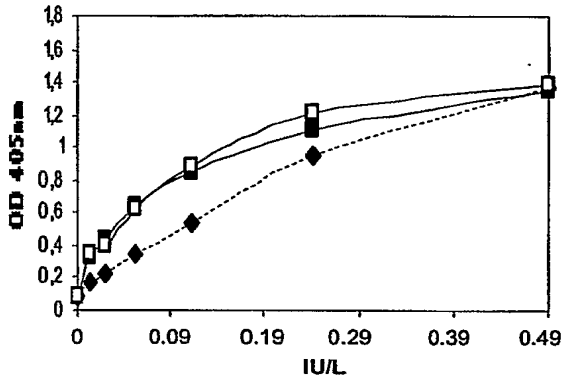


Figure 4A



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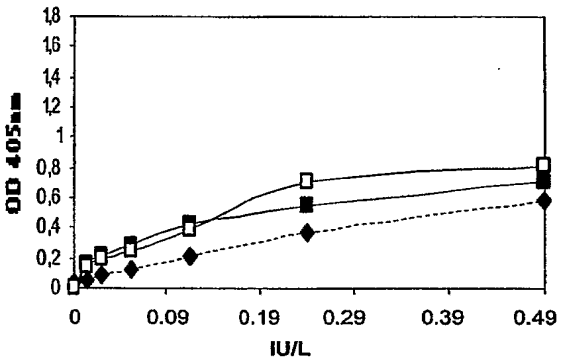

Figure 4C

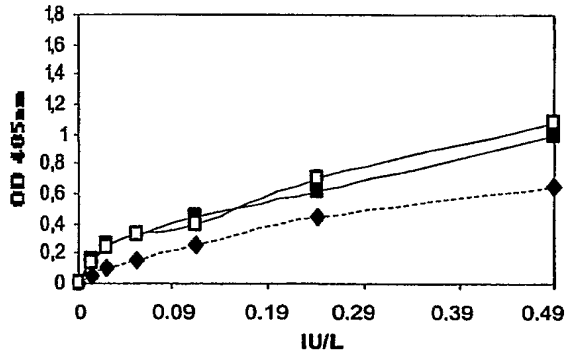

Figure 4D

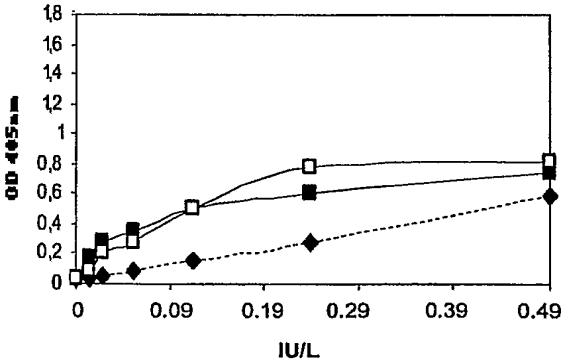

Figure 4E

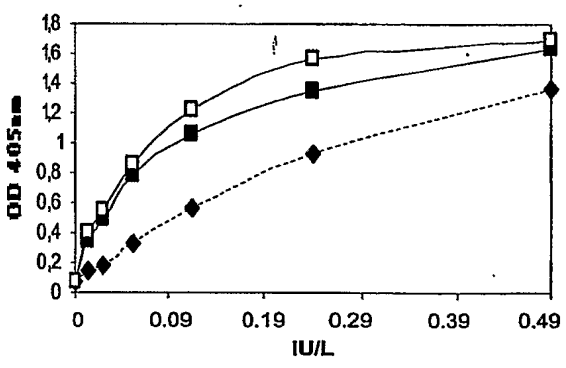

Figure 4F

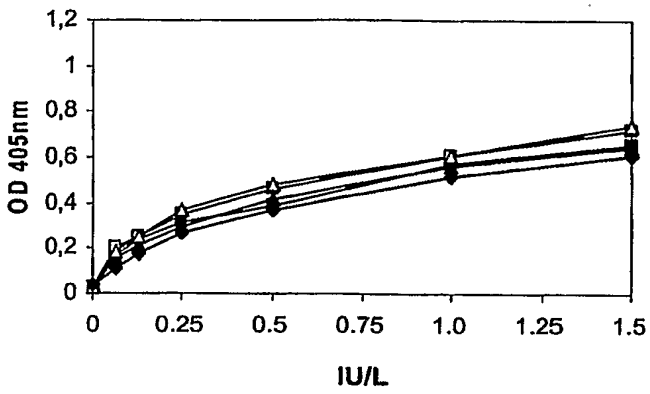

Figure 5A

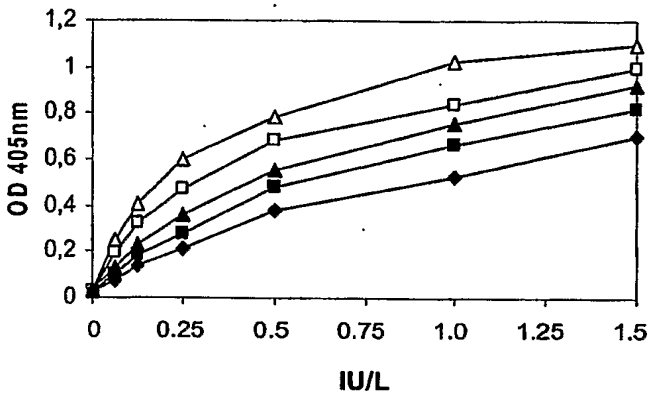

Figure 5B

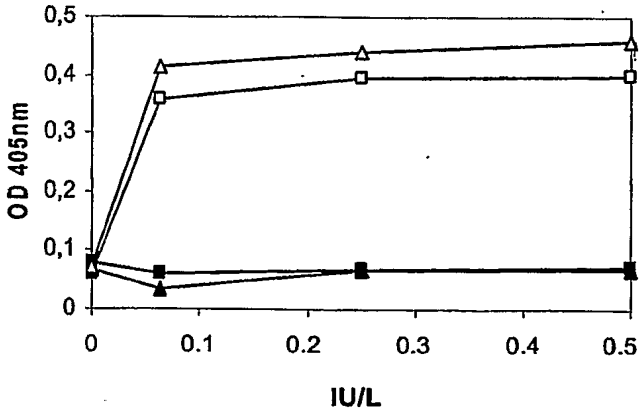

Figure 5C

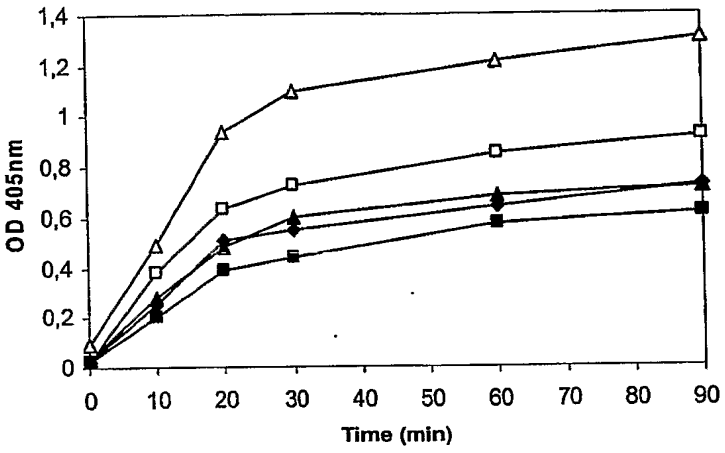

Figure 6A

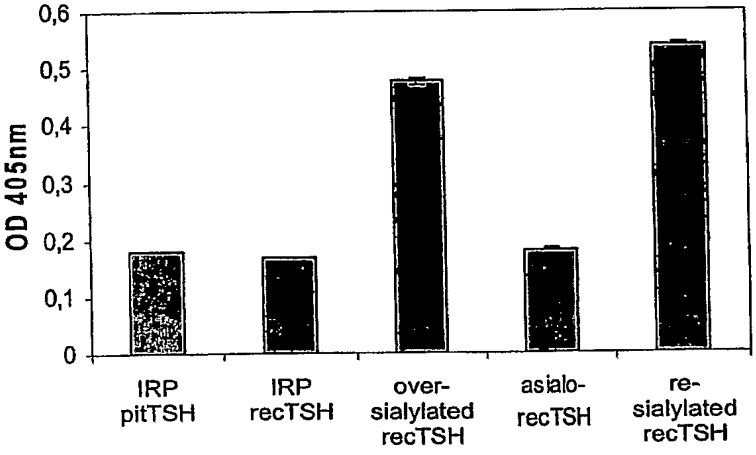

Figure 6B

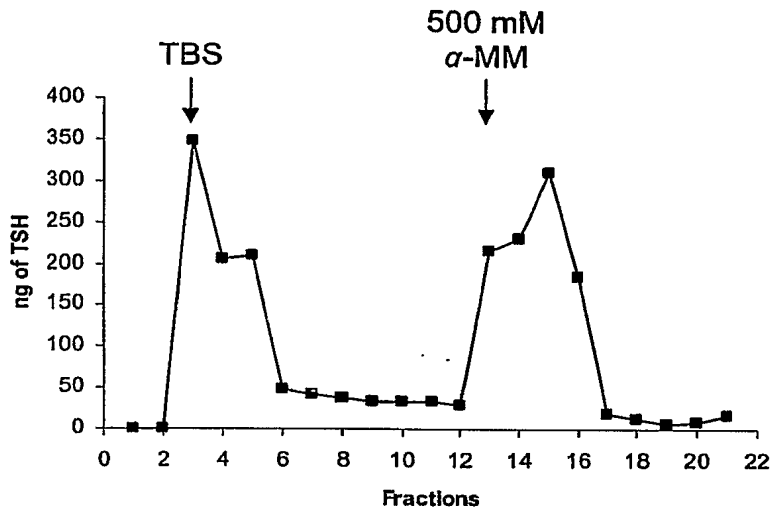

Figure 7A

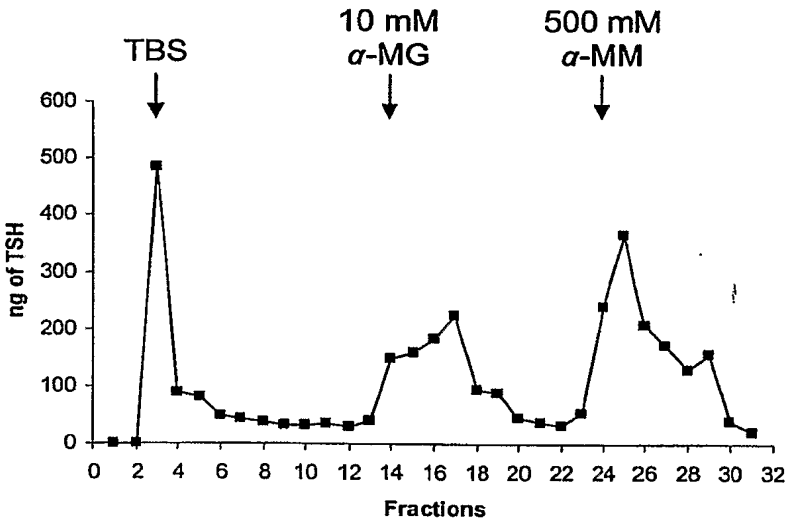

Figure 7B

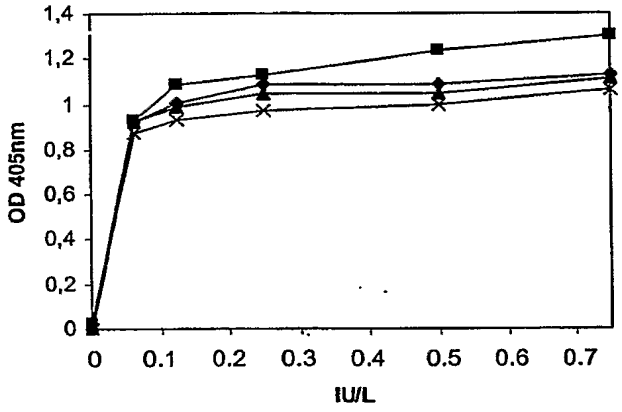

Figure 8A

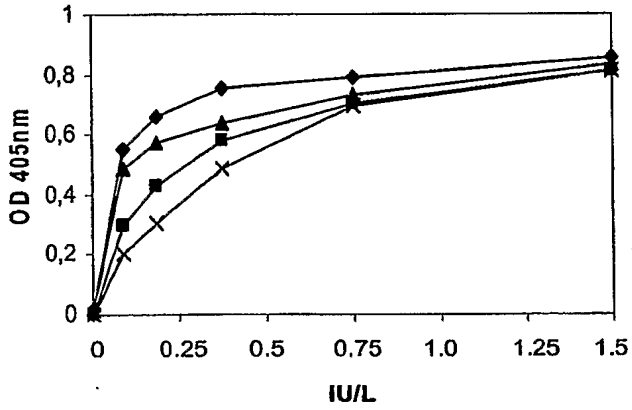

Figure 8B

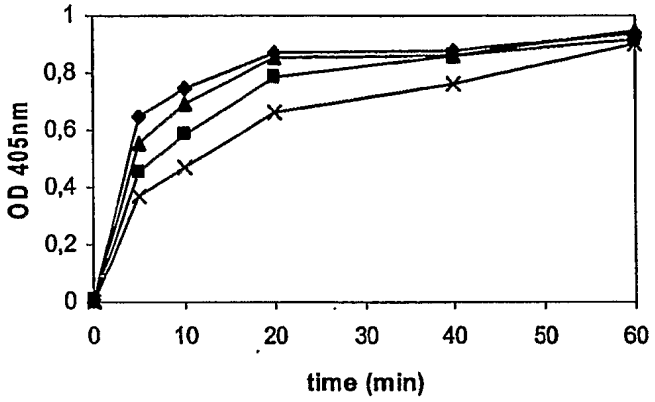

Figure 9A

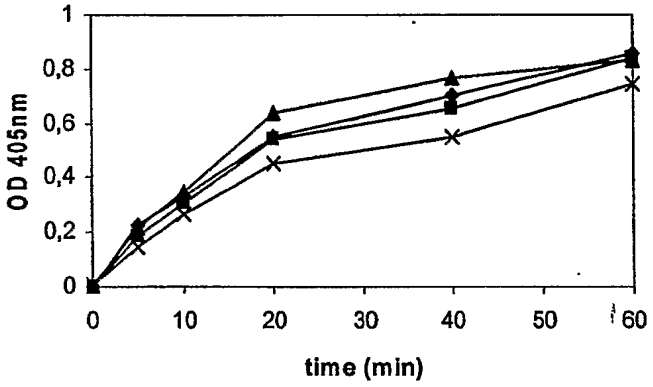

Figure 9B

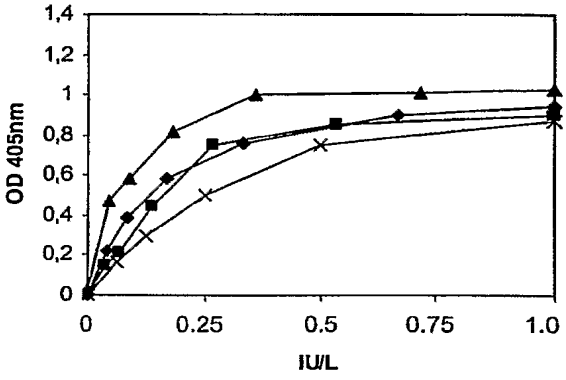

Figure 10A

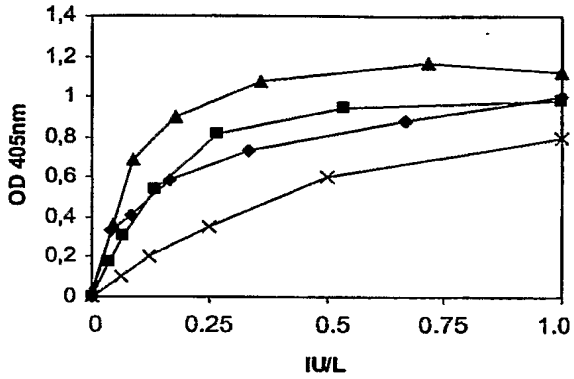

Figure 10B

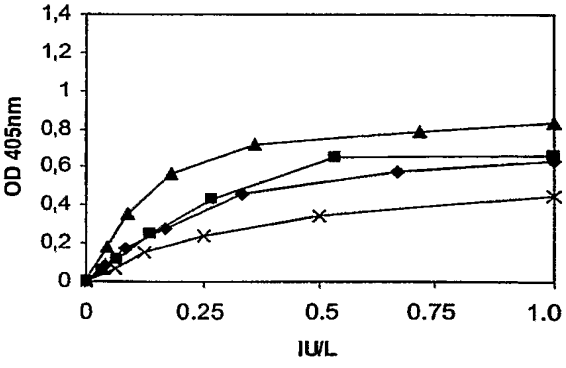

Figure 10C

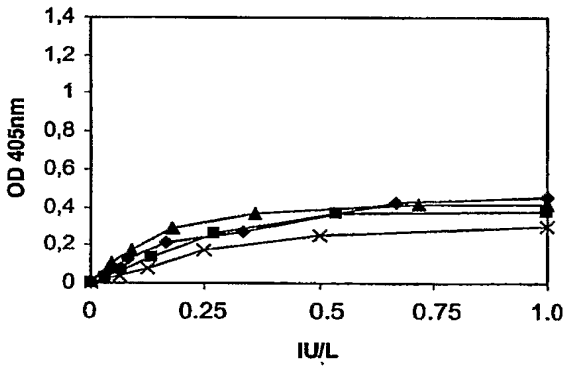

Figure 10D

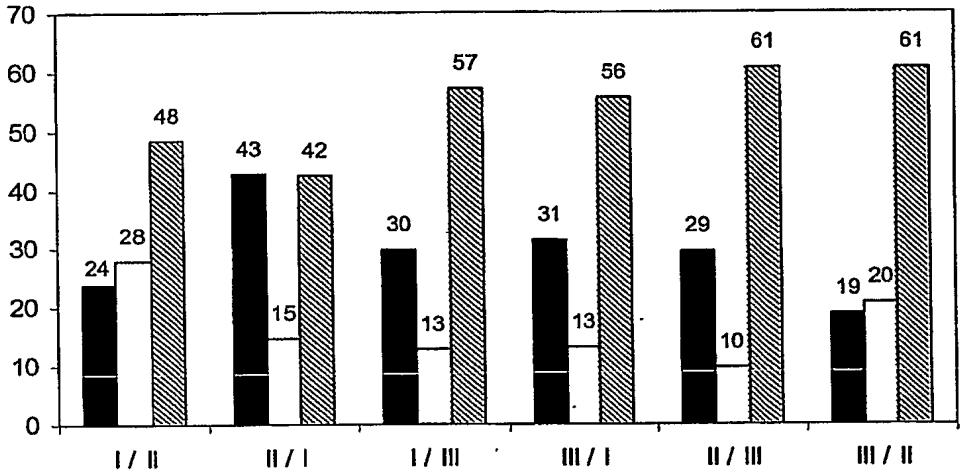

Figure 11A

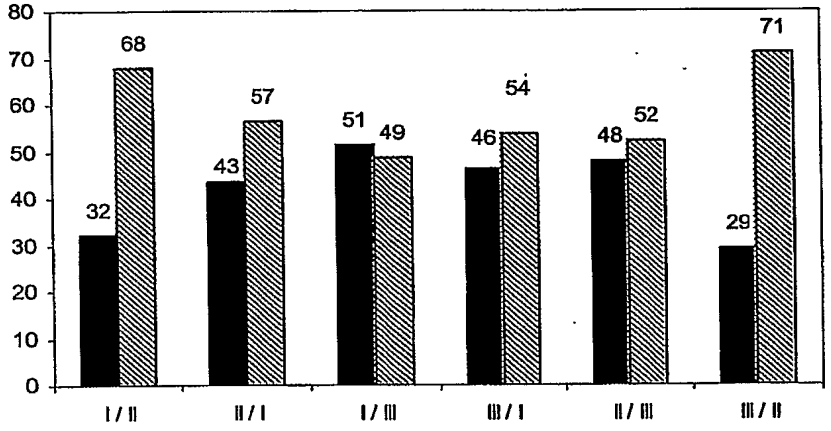

Figure 11B

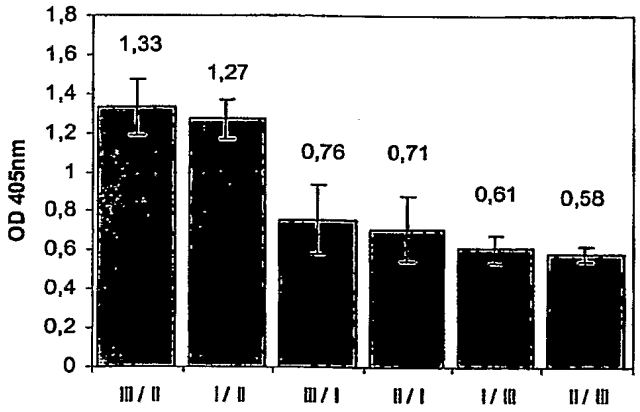

Figure 12A

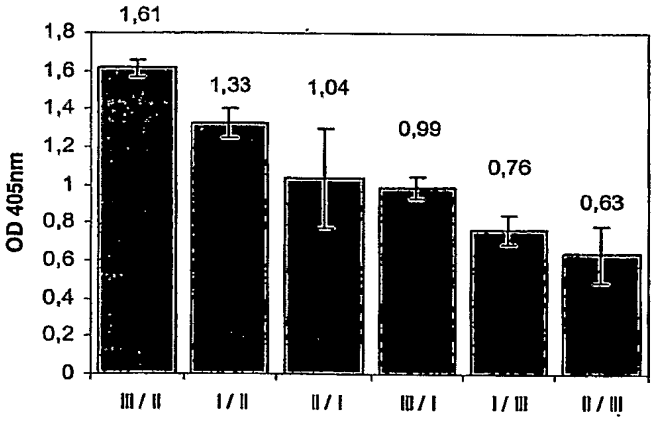

Figure 12B

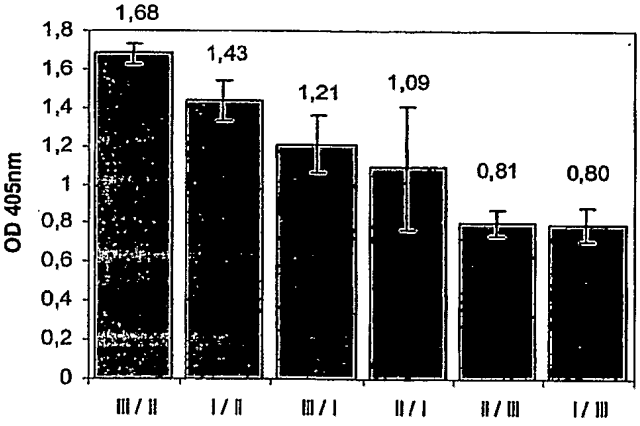

Figure 12C

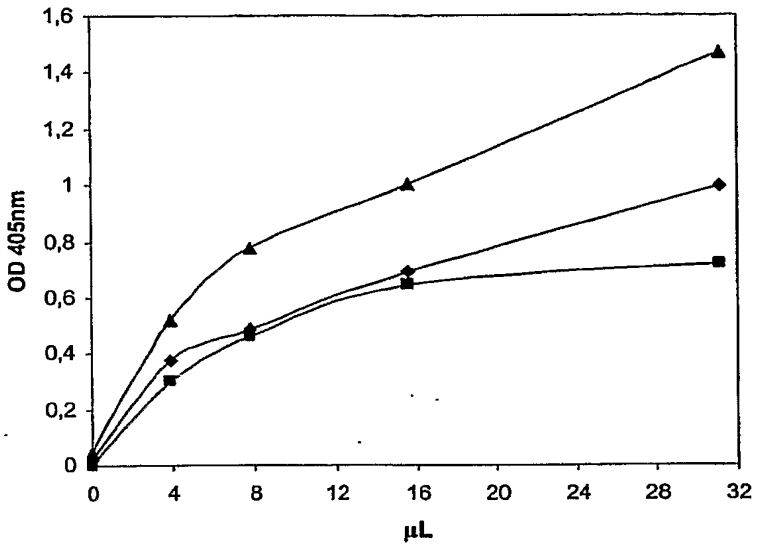

Figure 13A

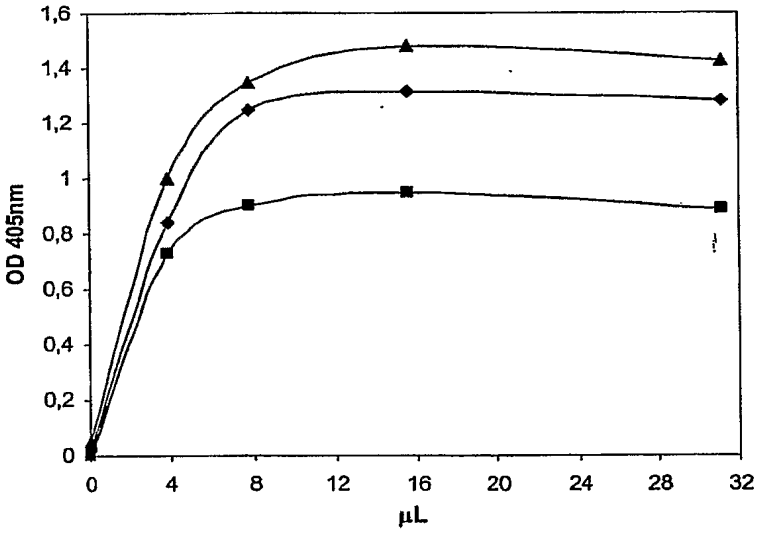

Figure 13B